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PRINCIPAL INVESTIGATOR: Juan Mendez, Ph.D.

Dr. Bruce Stillman

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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6. AUTHOR(S)
Juan Mendez, Ph.D.
Dr. Bruce Stillman

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

E-MAIL:
mendezj@cshl.org

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The coordination between cellular DNA replication and mitosis is critical to ensure controlled cell proliferation and accurate transmission of the genetic information as cells divide -two aspects of cellular life tipically lost in cancer.
In order to unravel the molecular mechanisms of human DNA replication in normal and cancer cells, we have started a search for human DNA sequences that serve as "replicators", this is, binding sites for human proteins involved in the initiation of DNA replication. Preliminary results are presented.

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Introduction

Most human cells proliferate by mitosis, i.e., each cell divides itself to give rise to a pair of identical daughter cells. Before mitotic division, all the DNA contained in the different chromosomes is duplicated, in order to pass one exact copy of the genetic information to each of the newborn cells. The DNA replication process has to be faithful, to prevent accumulation of mutations, and properly timed within the cell division cycle, to ensure that cells do not divide before the entire DNA has been copied. Understanding the molecular basis of this coordination of DNA replication and mitosis in human cells is necessary to understand cancer, the vast group of diseases that originate when cells divide without control. Within the past few years, several human genes required to regulate the initiation step of DNA replication have been identified and several origins of bi-directional replication (OBRs) have been mapped within 0.5-2 kb in different regions of the genome[1,2]. Our goal is to identify human replicator sequences by immunoprecipitation of DNA-bound initiator proteins.

Body

During the first year of research, we have optimized an experimental protocol for *in vivo* crosslinking of proteins to DNA in tissue culture human cells. In brief, cells were treated during 10 min with the crosslinking agent formaldehyde (at a concentration of 1%). The crosslinking treatment was quickly stopped by addition of 0.125 M glycine to the media, and the cells were washed and collected for extract preparation. Nuclear extracts were then sonicated in order to fragment the crosslinked chromatin in small molecules, ranging from 500 bp to 2 Kb in size. The different steps in the overall process –time length of the incubation with the crosslinking agent, ionic strength and detergent composition of the extract buffer, and sonication time- were optimized in a series of pilot experiments. Chromatin prepared in this way was ready for immunoprecipitation assays.

Variations of this technique have been used successfully to identify *in vivo* binding sites of a variety of proteins, including replication factors in yeast[3-5]. Because this is a complex experimental design, we first tested it using antibodies directed against a transcription factor (SNAPc190) whose binding site in human chromatin is known[6] and can be identified specifically by chromatin immunoprecipitation after crosslinking[7]. Using the experimental procedure outlined above, we reproducibly obtained an 8- to 10-fold enrichment in the SNAPc-190 binding site versus a distal site located 2 Kb upstream in the DNA. This result served as a positive control for our experimental approach.

Even though human DNA replication starts from several thousand different origins, barely twenty of them have been identified to date, and only three have been characterized in detail[2,8]. In a first approach, we have tried to identify replicator sequences in the human β-globin locus. This region is fully sequenced and contains a bi-directional origin of DNA replication[9-10]. Eleven sets of oligonucleotide pairs were designed across the region to amplify fragments of aproximately 400 bp by PCR (Figure 1). Using these primer pairs and purified genomic DNA as a template, unique sequences within the β-globin region were amplified by PCR with comparable efficiencies.

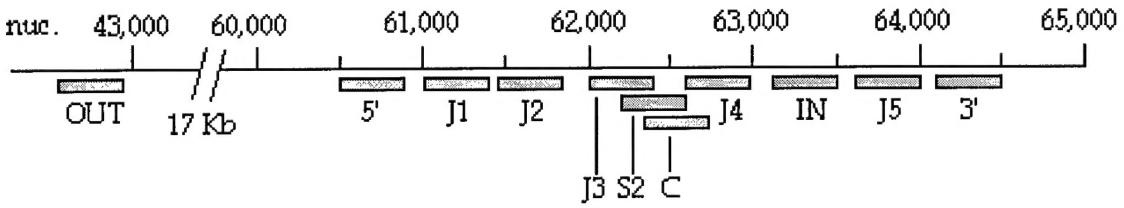

Figure 1. Different fragments of the β-globin locus were amplified with specific primer pairs. Primer pair "OUT" was located 17 Kb upstream of the main locus. The DNA molecules amplified by primer pairs "J3", "S2" and "C" are partially overlapping. In two immunoprecipitation experiments with anti-hOrc2p antibody, the DNA fragment amplified with primer pair "S2" was enriched 2-fold relative to any other fragment. See body text for details.

Several immunoprecipitation experiments have been attempted using a monoclonal antibody to hOrc2p, the second largest subunit of the human origin recognition complex. This protein is stably associated with chromatin during the cell cycle[11]. The goal of the experiment is to use hOrc2p-immunoprecipitated DNA as template for PCR reactions with the 11 primer pairs described in Figure 1. Quantitation of the different amplified products would indicate whether hOrc2p is preferentially bound to a specific DNA sequence within this region. In two independent experiments, we detected a slight (2-fold) enrichment in a DNA fragment corresponding to nucleotides 62,202-62,597 (primer pair S2). However, this enrichment is very small and could be due to experimental variations. We are currently assessing the reproducibility of this observation. We have not detected other specific enrichment of any particular fragment within the β-globin region.

There are several possibilities to explain the lack of strong enrichment in a particular DNA sequence in this locus. From a technical point of view, the efficiency of the immunoprecipitation step after crosslinking might be too low for our current detection limits. This problem could be addressed by a scale-up of the experimental protocol. Alternatively, the epitope in hOrc2p could be masked after the crosslinking step, making it inaccessible to the antibody. To overcome this limitation, a broader range of cellular targets could be used, including other hORC subunits, hCdc6p and hMcm proteins. We have recently found that in human cells, Cdc6p is tightly associated with chromatin during most of the cell

cycle, and the Mcm proteins associate with chromatin specifically at late mitosis and remain on chromatin during G1 and until the onset of S phase[11]. Based on previous studies in yeast, it is very likely that human Cdc6p and Mcm proteins will be complexed with the chromatin at the replication origins, and somehow "license" them to initiate DNA synthesis. Besides, we have recently raised and characterized a battery of new polyclonal and monoclonal antibodies directed against human Cdc6 and each of the six human Mcm proteins. These antibodies are highly specific and can immunoprecipitate the target proteins from cell extracts[11]. The use of antibodies directed to hCdc6 and hMcm proteins, in addition to hORC, will help to minimize the risk of epitope masking.

On the other hand, the fact that there is a *bona fide* replication origin within the β-globin lucus does not necessarily guarantee that it contains hORC binding sites. Actually, initiation of DNA replication at this site is modulated by a locus control region located several Kb upstream of the actual origin of replication[12]. To cover the possibility of the β-globin locus not containing actual hORC binding sites, we plan to extend our analysis to another known origin of replication in human cells, located downstream of the lamin B2 gene[2,8]. The sites of bidirectional DNA replication have been mapped at the nucleotide level, and genomic footprinting experiments revealed a region of protein-DNA interactions very close to the initiation site[13]. The nature of these proteins is unknown, but hORC, Cdc6p and Mcm proteins are very good candidates.

Finally, another useful variation that we will consider will be to carry out similar experiments with cells synchronized in the G1 phase of the cell cycle by centrifugal elutriation. This additional step will select cells in which pre-replicative complexes are assembled at the origins of replication, and it will be particularly useful when using anti-Mcm antibodies, because after initiation, the Mcm proteins can move with the replication forks[4] perhaps functioning as a replicative helicase[14].

Key research accomplishments:

- Optimization of an experimental procedure to succesfully immunoprecipitate DNA sequences after *in vivo* protein-DNA crosslinking. Confirmation of the validity of this method by immunoprecipitation of specific DNA binding sequences of a known transcription factor (SNAPc-190).

- Confirmation that human initiator factors such as hOrc1p, hOrc2p and hCdc6p are bound to chromatin during the cell cycle. hMcm proteins are bound to chromatin during late mitosis, G1, and early S phase, making all these proteins good targets for chromatin immunoprecipitation experiments.

- Design of multiple oligonucleotide pairs that serve as primers to amplify different DNA regions within the human β-globin locus, and optimization of the amplification reactions.

Reportable outcomes:

-Communication to the Cold Spring Harbor Laboratory Meeting on "The cell cycle" (May 2000). Abstract follows:

REGULATION OF HUMAN ORC1, CDC6 AND MCM PROTEINS DURING THE CELL CYCLE

Juan Méndez, X. Helena Zou-Yang and Bruce Stillman. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724.

Evidence obtained from yeast and *Xenopus* indicate the initiation of DNA replication to be a multistep process. The origin recognition complex (ORC), Cdc6p and minichromosome maintenance (MCM) proteins are required for establishing pre-replicative complexes, upon which initiation is triggered by the activation of cyclin-dependent kinases (CDKs) and the Dbf4p-dependent kinase Cdc7p. The identification of human homologues of these replication proteins allows investigation of S phase regulation in mammalian cells. Using centrifugal elutriation of several human cell lines, we demonstrate that whereas hOrc2p and hMcm protein levels are constant throughout the cell cycle, hOrc1p and hCdc6p levels vary. hCdc6p is almost absent in early G1 and accumulates until cells enter mitosis. hOrc1p levels, on the other hand, decrease after the G1/S transition. Both proteins can be polyubiquitinated *in vivo* and are stabilized by proteasome inhibitors. $p45^{Skp2}$, a subunit of the ubiquitin-protein ligase SCF that is required for S phase progression, is involved in the proteolysis of hOrc1p. Because hOrc1p is likely the critical ATPase subunit of hORC, this result suggests a novel cellular mechanism by which re-initiation of DNA replication is prevented. Using biochemical fractionation of human cells, we show that a fraction of hCdc6p is present on chromatin throughout the cell cycle, whereas hMcm proteins alternate between soluble and chromatin-bound forms. Loading of hMcm proteins onto chromatin occurs in late mitosis concomitant to the destruction of cyclin B, suggesting that the mitotic kinase activity inhibits pre-replicative complex formation in human cells.

Conclusions

1. An experimental protocol has been designed and optimized to immunoprecipitate chromatin-bound human proteins.

2. This protocol has been validated using a human transcription factor with a known DNA-binding sequence, and it is currently being used to try to identify human replicator sequences, using antibodies to several human initiator factors (hORC, hCdc6, hMcm proteins).

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